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KINETIC TESTS OF MODELS FOR SUGAR TRANSPORT IN HUMAN ERYTHROCYTES AND A COMPARISON OF FRESH AND COLD-STORED CELLS

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We studied the time course of the entry of galactose into human erythrocytes from an external concentration of 500 mM, and analyzed the data by an integrated rate equation treatment. We found evidence for only a single, high-affinity site for sugar at the inner face of the membrane. We studied the effect of pre-loading cells with galactose at various concentrations on the entrance of galactose into the cell from 128 mM, and compared the result we found with a previous report of a similar experiment from 500 mM external sugar. We found no evidence of other than a high affinity for sugar at the inner face of the membrane. The data reject a model in which sugar transport occurs on two asymmetric, oppositely directed carriers. We studied exchange of glucose into and out of the cells as a function of sugar concentration, taking care to minimize metabolism of sugar. We found no evidence for other than a single component for glucose exchange. Our data reject the 'allosteric pore' model for sugar transport. The explanation of the high-affinity site for sugar at the inner membrane face thus remains enigmatic. We find a very significant difference in the kinetics of glucose exchange when we compare freshly drawn and long cold-stored blood. The K_m for exchange was almost twice as large for cold-stored as for fresh blood.

Introduction

The kinetic analysis of sugar transport across human erythrocyte membranes is still controversial. A particularly difficult problem has been the interpretation of the high-affinity site for sugar transport at the inner face of the cell membrane, found originally by ourselves [1] and later confirmed in a number of studies [2–4]. It is clear that many of the sugar transport data can be accounted for by the model of an asymmetric carrier possessing a high affinity for sugar at the outer face of the cell and a low affinity at the inner face [5,6]. But the high affinity site at the inner face is not so readily explained. Ginsburg [7] suggested that there are indeed two asymmetric carriers arranged back-to-back within the membrane, on the model of an asymmetric anti-parallel pair of carriers. He showed that such a model could explain many of the then available data. However, his model makes a very definite prediction: inhibition of net sugar entry on raising the concentration of sugar at the inner face of the membrane should show up two such inhibitory sites – a high- and a low-affinity site. Only the high-affinity site had been seen. We have now undertaken to test this prediction by an extended study of sugar entry under these, the so-called 'infinite-*cis*', conditions. We do not find evidence for the low-affinity site and hence we reject the Ginsburg model. Another explanation for the puzzling high-affinity site at the inner membrane face has been provided by Holman [8]. He proposes an 'allosteric pore' model in which the high-affinity site is a regulatory site and where there is negative co-operativity between sugar molecules bound to the subunits of a postulated oligomeric carrier. Holman's model also makes a

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definite prediction, in this case with respect to the results of an equilibrium exchange experiment, where sugar is present at both membrane faces at the same concentration and the movement of labeled sugar is followed. Holman predicts here complex kinetics in which both a high- and a low-affinity site for sugar movement should be seen. Holman et al. [9] have indeed presented evidence purporting to show the existence of such complex kinetics for the equilibrium exchange experiment. Since we had never seen such kinetics ourselves, although we had performed such experiments many times [10,11], as had other workers [5,12–16], we decided to re-investigate this problem introducing a number of variations in method which Holman et al. [9] emphasised were important in order to obtain the results they reported. Unfortunately, we have not been able to repeat their findings.

In the course of this work we have had occasion to compare equilibrium exchange uptakes in freshly-drawn and in cold-stored, outdated transfusion blood. We report on the significant differences we find in their ability to transport sugar.

Materials and Methods

Preparation of cells

Outdated transfusion blood was obtained from the blood bank and stored in the refrigerator until used (from 3 to 8 weeks). Fresh blood was obtained by venous aspiration into acid-citrate-dextrose and immediately processed. Before use, the blood was washed four times in a phosphate-buffered saline, each wash being for 20 min at 37°C, in order to remove all cellular glucose.

General procedures

Solutions used were: Phosphate-buffered saline: 147 mM NaCl/20 mM Na_2HPO_4. pH was adjusted to 7.4 with HCl. The total osmolarity was 310 mosmol/l. Conventional stopper solution: 1% (w/v) NaCl/10^{-6} M $HgCl_2$/1.25 mM KI/10^{-4} M phloretin. The stopper solution was held on ice, and stopped cells kept in ice for the few minutes that elapsed before centrifuging. Iodide-free stopper solution (used in the experiments of Table III only): 1% (w/v) NaCl/0.16 · 10^{-6} M $HgCl_2$/0.3 mM phloretin. This stopper was also held on ice before and during use. Radioactively labeled chemicals from Amersham International were the tritium-labeled compound for L-galactose and the ^{14}C-labeled compound for D-glucose. For measurements of sugar trapped within the cells, the sedimented cells were hemolysed in 4 ml water, an aliquot of 500 μl was taken for measurements of radioactivity, after precipitating protein with 50 μl 100% trichloroacetic acid, and an aliquot of 400 μl of the supernatant was taken for scintillation counting. Counts were converted to dpm by an external quench correction. The absorbance of the hemolysate was measured at 540 nm to determine the amount of red cells present in each sample before hemolysis. All data were calculated as dpm/absorbance at 540 nm. In most cases the absorbance at 540 nm was of the order of unity, corresponding to some 5 μl of intracellular water. This internal correction for recovery of red cells allowed us to pay strict attention to the timing of each assay, the volume of the red cells added being of little significance. We could allow also for losses of cells in centrifuging and the subsequent resuspensions.

Great care was taken in our experimental protocol to ensure that the different concentrations studied (where this was the variable) were assayed in random fashion, obviating any fear that a deviation from linearity in a plot of rates against concentration could arise from some artefact due to storing of the cells or to temperature changes.

All flux measurements were performed in a water bath at 20–21°C, with care being taken to minimize the period that centrifuge tubes containing cells or solutions were out of this water bath.

Net flux measurements

The time course of net influx of galactose from an external concentration of 500 mM was determined exactly as described by Ginsburg and Yeroushalmy [15], and the data analyzed according to Eilam and Stein [17]. Net influx of galactose from 128 mM external galactose into preloaded cells was performed essentially as in Ginsburg and Stein [2], except that the step of resuspension of labeled cells in di-*n*-butylphthalate was omitted. For each internal concentration of pre-loaded galactose, cells were allowed to accumulate labeled

sugar to equilibrium. The amount of this label divided by the appropriate galactose concentration was not statistically different for the different galactose concentrations. An average value was therefore used to calculate the individual equilibrium values, from which the fractional filling of the cells was derived. A linear regression of fractional filling against time gave the rate of net sugar entry. Uptake – nine time points in each case – was measured during the first 10 s in the case of internal galactose of 0.2 and 4 mM; over the period of 60 s for 8 and 16 mM sugar and over 5 min for 32 and 64 mM galactose. The mean internal galactose concentration was determined from the amount of label at the mid-time point at each galactose concentration, and corrected for the osmotic volume change of the cell, taking into account the additional 128 mosM galactose external to the cell.

Equilibrium exchange measurements

The method of Ginsburg and Yeroushalmy [15] was used with the following modifications. Cells were loaded with unlabeled glucose (for influx) or with ^{14}C-labeled glucose (for efflux) for the following times at 20°C: 0.5–4 mM, 2 min; 8 mM, 3 min; 32 and 64 mM, 20 min; at a 10% hematocrit. The cells were then immediately sedimented in a refrigerated centrifuge and then kept on ice during the few minutes that it took to obtain a complete time course, each concentration of glucose being separately handled. For the flux measurements, 20 μl of the packed cells were rapidly vortexed into 2 ml incubation mixture containing glucose at the same concentration as the loading solution, but labeled with ^{14}C for the influx points, or unlabeled for the efflux points. At the desired times (measured by means of a metronome for times less than 30 s) ranging from 4 s to 20 min, 8 ml of a mercury stopping solution was added to terminate uptake. The cells were thereafter handled as described for the net entries from 128 mM galactose above. The fractional filling of cells was determined by measuring the uptake of label (at zero times for efflux measurements, or at 2–20 min, according to concentration as above, for influx experiments), and taking the mean of such values over all glucose concentrations to be the equilibrium value of label uptake. (For representative data see Table I). From the value at each time point, including the equilibrium values, we subtracted the amount of label trapped between the cells, this being determined by measuring label taken up at zero time (for influx) or at long times (for efflux) (see also Table I). At each glucose concentration we followed the time course of equilibration of label for, in general, seven time points. The rate constant for exchange of label was found [17] as the mean of these seven values for the computed functions $(\ln(f))/t$ for efflux (see Table II) or $\ln(1-f)/t$ for influx, where f is the fractional filling of the cells at time t. Data are reported as the mean of such estimates of the rate constant together with the derived standard error of the mean. The average S.E. was 7%.

Inhibition by glucose of 3-*O*-methylglucose influx in equilibrium exchange conditions was measured exactly as for glucose exchange influxes, except that sugar influx was determined at one time point, in triplicate, at each glucose concentration. The uptake times were as follows: for 0.2 and 4 mM glucose, 5 s; for 8 mM, 6 s; for 16 mM, 8 s; for 32 mM, 10 s; for 64 mM, 12 s. Fractional filling of the cell by 3-*O*-methylglucose was calculated by use of values for uptake of label at zero time and at 2 min, no glucose being present. The average of the S.E. for this experiment was 7%.

Results

Infinite-cis experiments

We have previously [2] described two methods by which the kinetic parameters of net sugar entry into erythrocytes can be obtained. In the first method (Fig. 1a,b) one measures the time course of sugar entry into the cells over an extended time scale. In Fig. 1, we depict the time course of galactose entry into initially sugar-free human erythrocytes from an external concentration of 500 mM. The slope of this uptake curve at any time is the instantaneous rate of sugar uptake. This slope changes markedly as sugar enters the cell and, presumably, binds to the sugar binding site at the inner face of the membrane. As can be seen even by a cursory inspection of the data on the expanded time scale, the initial rate of sugar entry is reduced to something like one-half when f, the fractional filling of the cell, is of the order of 0.1,

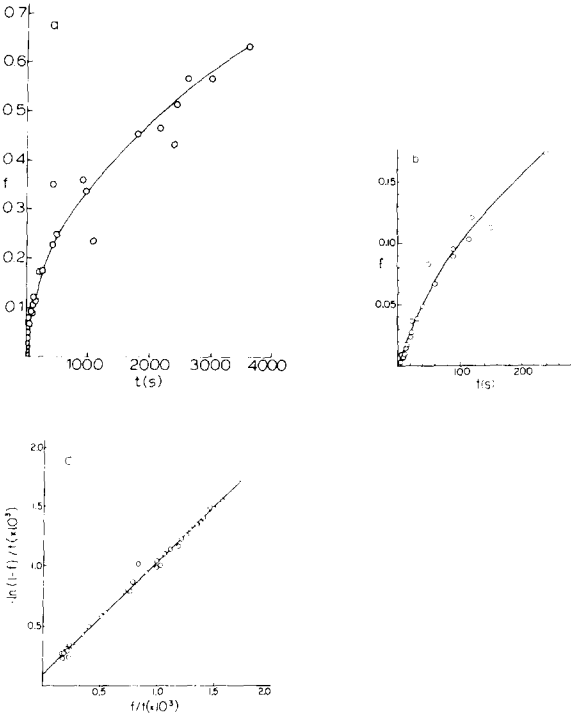


Fig. 1. Net entry of galactose into human erythrocytes at 20°C from an external concentration of 500 mM. The fractional filling of the cells, f, is calculated as ((dpm/absorbance at time t)−(dpm/absorbance at $t=0$))/((dpm/absorbance at equilibrium)−(dpm/absorbance at $t=0$)). The data are represented on an expanded time scale in b, and in the form of a semi-logarithmic plot (see text) in c. The line in c is drawn by linear regression through the points, the curves in a and b have no theoretical significance.

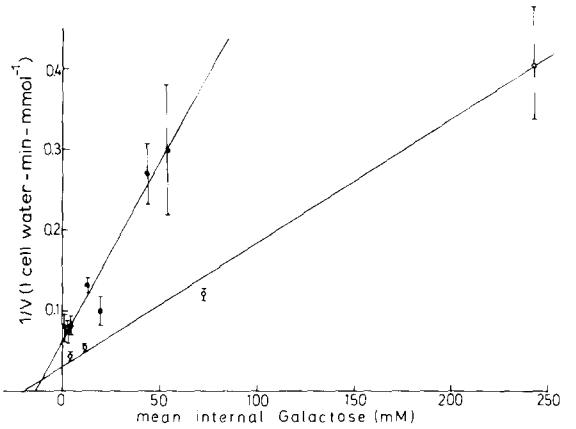


Fig. 2. An infinite-*cis* experiment. Reciprocal of the rate of uptake of galactose, ordinate, into erythrocytes at 20°C, as a function of the internal galactose concentration during the course of uptake, abscissa. The bars represent ±S.E. The upper line (●———●) is the regression line $y = 0.0578 \pm 0.0129 + (0.00449 \pm 0.00047)x$, through the points for an external concentration of 128 mM galactose. The derived half-saturation concentration for internal galactose is 12.86±3.17 mM. The lower line (○———○) is for data taken from Ginsburg and Stein [2] for an external galactose concentration of 500 mM. The regression line is $y = 0.0309 \pm 0.0117 + (0.001543 \pm 0.000091)x$. The derived half-saturation concentration for internal galactose is 20.10±7.69 mM.

i.e., near 50 mM galactose. We have shown [2,17] that an integrated rate equation treatment of such uptake data requires one to plot the function $(\ln(1-f))/t$ against f/t, where f is the fractional filling of the cell at time t. A simple single site at the inner membrane face will lead to a linear plot of these functions. The initial velocity of sugar uptake is given by $(y\text{cept}/(1-m)) \cdot S$, while the concentration of sugar within the cell at which the net uptake is reduced to one-half this initial rate is $(S^2(1/m-1))/P$, where $y$cept is the y intercept and m the slope of such a plot, while S is the external sugar concentration and P is the osmolar concentration of impermeable matter within the cell. Fig. 1c shows the required semilogarithmic plot of the data of Fig. 1a. The 33 time points on this curve cover a 1000-fold range of times and a 100-fold range of internal sugar concentrations. There is no hint of any deviation of these points from a simple straight line, and hence no evidence for the two sites of sugar binding that the Ginsburg [7] model would required to be present at the inner membrane face. According to these data, galactose enters the cell with an initial velocity here of 0.99 ± 0.27 mmol/l cell water per min. The uptake is one-half this initial rate when the internal sugar concentration is 40.9 ± 10.5 mM.

To confirm this finding, we used the alternative procedure, which is to preload cells with sugar at defined concentrations and then to determine how this internal sugar reduces subsequently measured net uptakes. Fig. 2 presents our data where we plot the reciprocal of the initial rates of net galactose entry into pre-loaded cells, as a function of internal galactose from (upper curve) an external concentration of 128 mM. There is no evidence of anything more than a simple single site for sugar at the inner face, in that a linear plot is quite compatible with our data. If there were any hint

that two lines were a better fit, they would be two lines bent convex to the x-axis. The Ginsburg model [7] would require two intersecting lines bent concave to the x-axis. Moreover, when we compare our new data with those from an external galactose concentration of 500 mM, reported previously by Ginsburg and Stein [2] and replotted as the lower curve of Fig. 2, we find that the half-saturation concentrations for internal galactose, derived from the intercepts on the abscissae of these data, are 12.9 ± 3.2 mM and 20.1 ± 7.7 mM for external galactose of 128 mM and 500 mM, respectively. These estimates of the half-saturation concentrations are not very different from one another but, in particular, the value appropriate to the lower galactose concentration is not higher than that from the higher, 500 mM, galactose.

Equilibrium exchange experiments

In the next set of experiments we used glucose as the test sugar, loading the cells to equilibrium with unlabeled sugar at concentrations over the range 0.5 to 64 mM, and then studied the influx or eflux of ^{14}C-labeled sugar.

Holman et al. [9] suggested that results of previous workers might have been in error as a result of metabolism of labeled glucose during the prior loading of labeled sugar for efflux experiments. To minimize the effect of metabolism we took care to load the cells with sugar for the minimum time necessary to achieve equilibrium with label at each glucose concentration (2 min at room temperature for the lowest glucose concentrations rising to 20 min for 32 and 64 mM glucose) and we took care to standardize this time, at any sugar concentration, in our series of experiments. Table I records the uptake of label at each glucose concentration at zero time and at the end of the experiment (2 min for the lower glucose concentrations rising to 20 min for the higher) in an efflux experiment (columns 2 and 3), and in an influx experiment (columns 4 and 5). The uptake of labeled glucose at zero time for efflux experiments and at the end of the equilibrium time for influx experiments does not depend on the glucose concentration, showing that everywhere equilibrium had been achieved. In addition, the cells lose glucose uniformly at all concentrations in the efflux experiment, showing that insignificant amounts of label

TABLE I

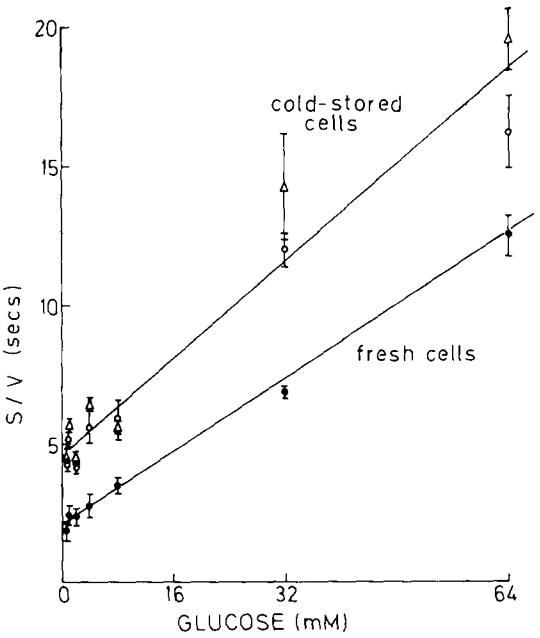
ZERO-TIME AND EQUILIBRIUM LEVELS OF D-[^{14}C]GLUCOSE IN ERYTHROCYTES FOR AND EFFLUX AND AN INFLUX EXPERIMENT, AT DIFFERENT GLUCOSE CONCENTRATIONS, AT 20°C

Cells were pre-equilibriated with labeled glucose (for efflux) or with unlabelled glucose (for influx) at the various concentrations listed in column 1, as described in the text. The cells were subsequently exposed to glucose at these same concentrations, but now either unlabeled (for efflux) or labeled (for influx). Uptake was terminated at zero time or at equilibrium which was taken to be 2 min for glucose at 0.5–4 mM, 3 min at 8 mM and 20 min at 32 and 64 mM. Each value recorded is a mean of duplicate determinations. The radioactively labeled glucose counted at 1295 dpm/μl solution for the efflux experiment and at 641 dpm/μl for the influx experiment.

Glucose concn. (mM)	Cell content of D-[^{14}C]glucose (dpm/absorbance at 540 nm)			
	During efflux of label		During influx of label	
	Zero time	At equilibrium	Zero time	At equilibrium
0.5	875	40	6	448
1	907	51	4.5	448
2	909	28	2	509
4	877	29	2	436
8	874	27	3	439
32	905	33	13	511
64	881	33	6	440
Mean ± S.D.	890 ± 16	34 ± 8	5 ± 4	462 ± 33

are metabolized into a form trapped within the cell. (The table provides some estimate, too, of the accuracy of our technique. The standard deviation of the estimates of the uptake of label at zero time in an efflux experiment is here some 2%, each estimate being derived from the mean of a duplicate assay. In the influx experiments the standard deviation of the estimates of the equilibrium uptakes was here some 7%.)

We checked whether efflux of sugar was indeed exponential over the whole of the time course of an experiment. Table II gives the values of the function f and $\ln(f)$, where f is the fractional filling of the cell with sugar at various times of efflux, for cells pre-equilibriated with 1 mM and 50 mM glucose. We also list the value of $(\ln(f))/t$,



at each time t. If all the sugar is flowing out according to single-compartment kinetics, this ratio should be invariant with time. The data demonstrate no lack of accord with single-compartment kinetics. We need not assume that sugar efflux is affected by metabolism of some of the sugar or by anomeric interconversion of α- and β-sugar iso-

Fig. 3. Equilibrium exchange of glucose at 20°C. The ordinate is the reciprocal of the rate constant of sugar exchange, measured at the equilibrium sugar concentration given on the abscissa. The upper line is the regression line through data obtained using 8-week cold-stored blood. It is $y = 4.647 \pm 0.401 + (0.2184 \pm 0.0147)x$. The derived half-saturation concentration for glucose is 21.28 ± 2.33 mM. The triangles are points for an influx experiment, the open circles are for an efflux experiment. The lower line (solid circles) is the regression through data obtained for an influx experiment with freshly drawn blood. The regression is $y = 2.063 \pm 0.133 + (0.1627 \pm 0.0045)x$. The derived half-saturation concentration is 12.68 ± 0.89 mM. The bars represent ± 1 S.E.

TABLE II

TEST OF EXPONENTIAL NATURE OF GLUCOSE EFFLUX IN AN EXCHANGE EXPERIMENT AT 20°C AT 1 AND AT 50 mM GLUCOSE

The blood used was 6-week-old cold-stored transfusion blood. Experimental method as in text. The fractional filling, f, was calculated as ((dpm/absorbance at 540, at time t)−(dpm/absorbance, at equilibrium))/((dpm/absorbance at $t = 0$)/(dpm/absorbance, at equilibrium)). Each time point is the mean of a duplicate.

Glucose. concn. (mM)	Time of efflux (t) (s)	Labeled glucose content (as fractional filling (f)	$\ln(f)$	$\ln(f)/t$
1	3	0.555	−0.588	−0.196
	4	0.482	−0.729	−0.182
	5	0.361	−1.02	−0.204
	6	0.343	−1.07	−0.178
	8	0.214	−1.54	−0.193
				-0.191 ± 0.011 [a]
50	9	0.626	−0.468	−0.0520
	12	0.571	−0.561	−0.0467
	15	0.534	−0.628	−0.0419
	18	0.421	−0.864	−0.0480
	21	0.336	−1.09	−0.0519
	24	0.361	−1.02	−0.0425
				-0.0473 ± 0.0043 [a]

[a] Mean ± S.D.

TABLE III

COMPARISON OF THE EFFECT OF TWO DIFFERENT MERCURIC CHLORIDE-CONTAINING SOLUTIONS IN STOPPING GLUCOSE EXCHANGE ACROSS HUMAN ERYTHROCYTES: EFFLUX EXPERIMENT AT 20°C

The experiment used 8-week cold-stored transfusion blood, as in the upper curve of Fig. 3. The composition of conventional stopper solution (containing potssium iodide) and the solution used by Holman et al. [9] (without iodide) is given in Materials and Methods. Fractional filling, f, calculated as in Table II and text. Each rate constant is derived from a time course of six intermediate time points, with a zero time and equilibrium time measured in addition. Values are means ± S.E.

Equilibrium glucose concn. (mM)	Rate constant of glucose efflux as $\ln(f)/t$ (s^{-1}) Mean ± S.E. ($n = 6$)	
	Conventional stopper solution	Stopper solution of Holman et al. [9]
1	0.207 ± 0.015	0.219 ± 0.013
4	0.160 ± 0.016	0.170 ± 0.006
32	0.098 ± 0.005	0.110 ± 0.003

mers. Fig. 3 (upper curve) records our data on the efflux and influx of glucose for cold-stored erythrocytes, plotted as the reciprocal of the rate constant for sugar flux, as a function of the glucose concentration. There is no statistical difference between the two sets of data points (see legend to figure). Both efflux and influx of labeled glucose fit a simple one-site plot. We tested whether the mercury stopping procedure used by Holman et al. (which omitted the potassium iodide used [10] to carry mercury to the inner cell face) might be responsible for the difference between their reported findings and those we present in Fig. 3. Table III shows our data for equilibrium exchange of glucose at three concentrations obtained with our standard mercury stopping procedure and that used by Holman et al. [9]. We found no difference between the two sets of data, and the data we obtained were not statistically different from those reported in Fig. 3. The data from Fig. 3, from Table III and from another experiment in which influx and efflux were determined on the same cells on the same day were all combined together in a linear regression analysis. The data, 23 points, each derived from some eight time points, demonstrated a correlation coefficient of 0.965 and yielded a maximum velocity of sugar uptake of 4.82 ± 0.29 mmol glucose per litre cell water per s, and a half-saturation constant of 22.1 ± 1.9 mM. There was no evidence for any systematic deviation of the data points, with increasing sugar concentration, from the best-fit straight line.

The intercept on the ordinate in Fig. 3 gives the reciprocal of the rate constant for glucose entry at limitingly low sugar concentrations. The value found is 4.65 ± 0.40 s, quite different from the value of some 2.5 s that one could read off from the data reported by Holman et al. [9]. We were surprised at this discrepancy, as the value of this intercept seemed to be rather independent of whether the data fitted or did not fit a simple straight line. Thinking that perhaps the age of the cells might be responsible for this difference, we measured influx of labeled glucose in equilibrium exchange conditions, using freshly drawn blood. The data are presented as the solid circles in Fig. 3. The data clearly fit a very good straight line. The regression line here has a correlation coefficient of 0.9985 and yields a maximum velocity of uptake of 6.15 ± 0.17 mmol/litre cell water per s, with a half-saturation concentration of 12.7 ± 0.9 mM. The intercept on the ordinate here is 2.06 ± 0.13 s. Fresh and cold-stored human blood clearly display quite different transport kinetics, the major difference being in the almost doubled Michaelis constant, K_m, for sugar exchange in the cold-stored cells. We have found such a rapid uptake of sugar at low sugar concentrations for fresh blood from three donors (W.D.S., M.B.W. and also Professor H. Ginsburg) and the reduction of rate with cold-storing is a consistent finding. We found, too, that slowing down of galactose exchange was perfectly parallel to the slowing down of glucose exchange. In an experiment measuring glucose efflux from 1 mM we found a value for 1/(rate constant) of 2.0 s for freshly-drawn blood and of 3.5 s for 7-week-stored blood, while galactose efflux, also from 1 mM, was here 11 s and 19 s, respectively.

Holman et al. [9] reported that the inhibition by glucose of the influx of labeled 3-*O*-methylglucose also gave kinetics suggesting the presence of two affinity sites for glucose. Here again we attempted to reproduce such findings. Fig. 4 shows our data for the uptake of the glucose analog 3-*O*-methyl-

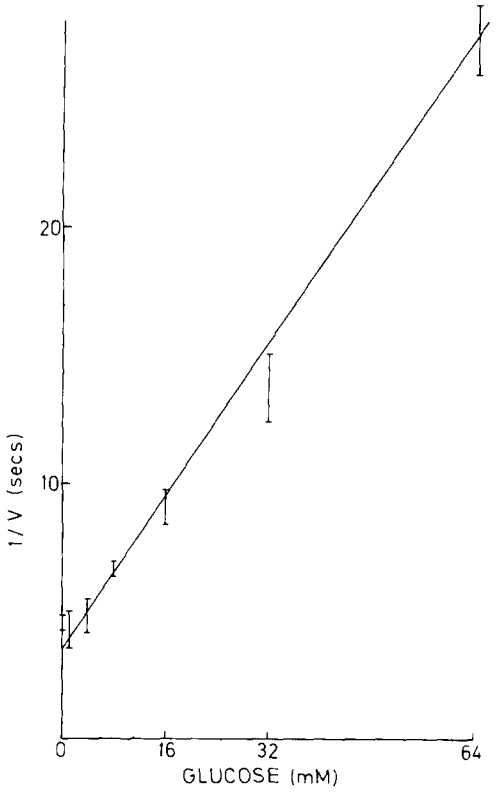


Fig. 4. Inhibition of the influx of 3-*O*-methylglucose into erythrocytes at 20°C, when cells were equilibriated with D-glucose before uptake measurements. The ordinate is the reciprocal of the rate of entry of 3-*O*-methylglucose as a function of the concentration of glucose (abscissa). Each point is given as ± 1 S.E. bar for a triplicate determination.

glucose (labeled with tritium) as a function of the glucose concentration with which the cells were pre-equilibriated. There is no sign in our data of anything other than a simple straight line fit to the data. Once again, if there were any hint that two lines were a better fit, they would be bent convex to the *x*-axis. A linear regression of these data gave a correlation coefficient of 0.993. The reciprocal of the rate constant for sugar entry was 3.62 ± 0.55 s, and the inhibition constant for glucose was 9.66 ± 1.55 mM, not very different from the value derived in Fig. 3 for glucose inhibition of entry of labeled glucose.

(Holman et al. [9] also report complex kinetics for the inhibition of fructose entry by glucose pre-equilibriated with erythrocytes. We have not attempted to repeat their findings in this case. 20 years ago, in unpublished experiments, one of us (W.D.S.) found that fructose and sorbose behaved differently towards inhibition by pre-equilibriated glucose. Sorbose uptake into erythrocytes appeared to be via a single component, inhibited in simple fashion by glucose. In contrast, fructose uptake appeared to be only partly inhibited by glucose (some 75%). It was assumed at that time that sorbose was, therefore, a better model substrate for the glucose carrier. All our subsequent work has therefore concentrated on sorbose to the exclusion of fructose.)

Conclusions

Our conclusions from this study are three in number. First, we confirm that net sugar transport into human erythrocytes is inhibited by a high-affinity site at the inner membrane face (Figs. 1 and 2). We find no evidence, however, for a low-affinity site, such as the two anti-parallel asymmetric carrier model of Ginsburg [7] would require. The Ginsburg model [7] would require a high- and a low-affinity site to be present, and this would appear in the plots of Fig. 2 as two intersecting lines bent concave to the *x*-axis. The data at low internal galactose concentrations would lie on a line intersecting the *x*-axis at low concentrations, while the data at high internal galactose would lie in a line cutting the *x*-axis far from the *y*-axis. In addition, sugar entry at lower external sugar concentrations would use preferentially the carrier possessing a high-affinity site at the external cell surface and hence be particularly inhibited by any low-affinity site at the inner surface. In contrast, when we compared inhibition of net sugar uptake from a high external galactose concentration (500 mM) with inhibition from a lower concentration (128 mM) we found (Fig. 2) no suggestion of any lower-affinity site with the 128 mM protocol. On the contrary, net galactose entry from high or lower external sugar seems to be inhibited uniformly by a single site of high affinity at the inner membrane face. The data reject the two anti-parallel asymmetric carriers model.

Second, we fail to find any complexity in the kinetics of glucose exchange into or out of human erythrocytes. Within the admittedly far from perfectly accurate data that we have obtained, only a

single site for sugar binding is seen, the same in influx or in efflux measurements of glucose exchange, and a similar, single site is seen for the inhibition of 3-*O*-methylglucose uptake by pre-equilibriated glucose. We have not found data lying on two lines concave to the *x*-axis, as the Holman [8] model would require. Our data display an average standard error of some 7% at each sugar concentration. Probably an accuracy at least an order of magnitude higher would be needed to resolve a two-component curve. We find no evidence for significant, kinetically detectable metabolism of glucose under the short incubation times (at room temperature) used, and there is no evidence that glucose exchange follows other than single compartment exchange kinetics. We cannot reproduce the findings of Holman et al. [9], although we have varied our technical procedures in accord with their prescriptions. We are at a loss to understand this discrepancy, but would wish here to point out that previous [5,10–16] studies of the sugar exchange in human erythrocytes have been in accordance with our present findings rather than with those of Holman et al. [9]. Since we cannot provide evidence for the two classes of sugar affinity that the allosteric pore model for sugar transport [8] would require, we are forced to reject this model also. The explanation of the high-affinity site for sugar transport present at the inner face of the cell membrane thus remains elusive and is the major enigma of the sugar transport kinetics in human erythrocytes.

Third, we find clear evidence that the kinetics of exchange transport of sugar are affected by the condition of the cells. We do not know of any previous work establishing this finding. It is the K_m for sugar transport that seems most affected by cold-storage of the cells, its value in long cold-stored blood being almost double that in freshly drawn blood (Fig. 2, upper versus lower curves). This finding might account for the variability previously noticed between different investigators' analyses of the transport kinetics. Thus, Eilam [16] noted that her data at low glucose concentrations were far different from those of Edwards [13], in the direction that might be expected from our present work if cells of different periods of storage were being studied. There are two preliminary reports in which transport systems in human erythrocytes vary their kinetic parameters with cell condition. Jarvis et al. [18] report that the uridine transport system of these cells behaves as a symmetric system in fresh cells but as a highly asymmetric system in cold-stored cells. In addition, the equilibrium exchange of uridine was somewhat faster into fresh cells and the system displayed a higher affinity in fresh cells, just as does glucose transport in our hands. At the lowest uridine concentrations, the reciprocal of the rate constant for nucleoside entry is 5.9 s for fresh blood and 11.2 s for cold-stored blood, a relation very similar to that which we find in Fig. 2. Jacquez [19] has reported that the maximum velocity of net sugar uptake into human erythrocytes is significantly increased when ATP-depleted cells have their internal ATP concentration restored. We have no comparable data on net uptakes and Jacquez has not yet reported data on sugar exchange, but it could well be that his findings and ours are related. Finally, it is very tempting to relate this finding in sugar transport kinetic parameters with the most interesting changes that White et al. [20] find on comparing strains of malignant and non-malignant fibroblast cells. With increasing malignancy comes an increased affinity for glucose in the affected cells. The state of the cell is a variable which clearly needs to be further explored in the analysis of the transport of sugars.

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